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(54) Title: ACTIVE PROBE AND TARGET DETECTION

(57) Abstract: A ribozyme is disclosed wherein the composition of the catalyst core sequence is comprised by two or more independent sequences and wherein catalyst activity is dependent upon the presence and association by hybridization of said components and a catalytically active conformation is assumed by hybridization with a target nucleic acid sequence comprising a sequence suitable for catalytic action. So long as essential and conversed bases are comprised by each of the individual, non-target strands, no sequence alone comprises the ribozyme or possesses catalytic activity. The target sequence of catalytic action and one or more of the sequences each comprising essential and conserved bases possessing catalytic activity may be consecutively associated. The ribozyme finds utility in both diagnostic and therapeutic applications. Generation of a surrogate template for direct detection or amplification is conditioned upon an active catalytic conformation of the ribozyme.

ACTIVE PROBE AND TARGET DETECTION

FIELD OF INVENTION

The present invention is in the fields of molecular biology, genetic engineering, and medical and veterinary diagnostics, and more particularly relates to the detection of nucleic acids. The invention relates to methods and compositions for detecting and quantifying the presence of specific target nucleic acid sequences. Target nucleic acid sequences of interest may be naturally or unnaturally derived and may be present in a variety of matrixes including biologic and non-biologic.

BACKGROUND OF THE INVENTION

This invention is directed to methods for detecting and amplifying desired nucleotide sequences. Recent advances in the general field of molecular biology have made it possible to detect specific genes of clinical and commercial importance. The use of nucleic acid hybridization assays as a research tool for the detection and identification of a unique deoxyribonucleic acid (DNA) sequence or a specific gene in a complete DNA, a mixture of DNA's, a mixture of DNA fragments or ribonucleic acid (RNA) sequences have made it possible to diagnose human disease at the genetic level.

The binding of molecules one to another is a basic theme of modern biology. General properties such as charge, hydrophobicity and Van Der Waal's forces have been utilized to characterize such molecular interaction. For example, polyanionic substances such as heparin are known to form strong interactions with polycationic substances. These interactions have been exploited for the *in vitro* isolation of molecules. Protein interactions have also been studied extensively. While proteins have been characterized by these properties globally, their content of amino acid residues comprising a wide diversity of functional groups and their arrangement into groups or domains within the molecule imparts properties of intermolecular interaction not readily apparent from assessment of these interactions as general properties.

High affinity interactions between molecules have received considerable attention. Generally, affinity and specificity of interaction are shared features. The

interaction of a protein hormone with its receptor is both specific and of high affinity. We now appreciate that these interactions are not passive. The docking of two molecules at a high affinity site is accompanied by conformational changes directly involving the sites of contact and extending to involve rearrangements at sites distant from the point of contact. Such rearrangements are thermodynamically stabilizing and result in the formation of high affinity interactions between the molecules.

Well-known examples are those between an antigen and its cognate antibody. Contact between the antibody and antigen result in conformational changes resulting in their stable interaction. Contact between an antigen and a non-cognate antibody does not result in a conformational change and a high affinity interaction does not occur. A comprehensive study performed by Jencks indicates that protein enzymes utilize binding energy derived from interactions with groups on the substrate not directly involved in the ultimate chemical transformation to stabilize the essential transition state (Jencks, W.P. Adv Enzymol (1975) 43:219-410).

Such interactions are also a common theme descriptive of the interaction of enzyme and substrate. The rearrangement induced by the specific interaction of the two molecules stabilizes the substrate in a transitional state between two chemical forms that, otherwise, are separated by an energy barrier. Where the interaction of two molecules directs the rearrangement of one molecule into a transition state between the two chemical states, the interaction is characterized as enzymatic. Our understanding of these fundamental molecular interactions has been extended from a recognition of interactions involving commonly understood properties, as described above, to now include an awareness that these interactions attain high affinity and specificity through mutually-induced, conformational changes extending throughout the interacting molecules. Such interactions involve the formation of secondary and tertiary structures. Recognition of antibodies with catalytic activity becomes understandable in the context of this paradigm.

Several features are now understood to distinguish global molecular interactions from high affinity interaction: First, we recognize affinity and specificity are related properties. As noted, only specifically interacting molecules generate conformational changes. Second, specific interactions provide much higher affinity interactions than expected on the basis of general properties. Third, molecular rearrangements induced

may favor a transitional state between two chemical states that are, otherwise, separated by an energy barrier unfavorable to transformation of a molecule between the two states. Moreover we have come to understand these properties as a result of the complexity and diversity available through the wide variety of functional groups provided by the amino acid residues of proteins. In contrast, nucleic acids, conventionally, have been thought to interact in more predictable ways through simple canonic interactions.

The discovery of RNA molecules unassociated with protein capable of catalytic modification of the phosphodiester bond in the laboratories of Sidney Altman and Thomas Cech has altered our simplistic view of nucleic acid interaction and has led to the development of synthetic, non-naturally occurring RNA molecules with enzymatic properties that permit their iterative processing of target sequences. The development of these engineered nucleic acid molecules was contingent upon recognition of a central and substantially conserved core structure and adjacent, mutable helical-duplex structures that contribute to alignment of the bases of the central core.

Most *cis*-acting, naturally occurring catalytic RNA's are not true enzymes. Where a true enzyme remains unaltered by participation in an enzymatic reaction and, therefore, may iteratively participate in subsequent reactions, most of the naturally occurring catalytic RNA sequences yet described are altered by their participation in a catalytic event and, thus, are not available for subsequent rounds of activity. Efforts to reengineer these naturally occurring sequences to function as true enzymes where the sequences comprising enzymatic activity and substrate are comprised on separate molecules have been successful. Chowrira in USP 5,631,359 and also Stage-Zimmerman et al. (Stage-Zimmerman, T.K., Uhlenbeck, O.C., *RNA*, (1998) 4:875-889) teach that these non-natural molecules comprise a catalytic core and adjacent target sequences configured to bind the target nucleic acid sequence through complementary Watson-Crick pairing in a conformation that brings the enzymatic portion of the molecule into close proximity to the scissile region of the target sequence. Once bound to the target, the enzymatic region acts to cut the target sequence. The hybridizing regions are so configured that they release the target fragments and the molecule is free to search for, bind and cleave new targets. Thus, recognition of the target nucleic acid sequence is mediated through complementary base-pairing within non-conserved portions of the

molecule that can be designed to form Watson-Crick pairing with a desired target, and once bound to the correct site, acts catalytically such as by hydrolysis of the target RNA.

The hammerhead ribozyme of Haseloff (USP 5,494,814), the hairpin ribozyme of Chowrira (USP 5,631,359) and the Tetrahymena rRNA intervening sequence catalytic RNA of Cech (USP 4,987,071) are examples of reengineered, naturally occurring catalytically active sequences that conform to the definition of a true enzyme. Unlike their *cis*-acting, natural counterparts, their substrate specificity becomes an important consideration in their design. Common to these engineered RNA's are regions that hybridize with the desired target sequence. Hybridization of these molecules to a substrate aligns the bases of the central core into an active conformation. Figure 1 shows a schematic representation of a hammerhead ribozyme (dashes represent Watson-Crick pairing.) Stems or helices are labeled I, II, and III according to the nomenclature of Hertel, K.J., Pardi, A., Uhlenbeck, O.C., Koizumi, M., Ohtsuka E., Uesugi, S., Cedergren, R., Eckstein, F., Gerlach, W.L., Hodgson, R. & Symons, R.H. (1991) *Nucleic Acids Res.* **20**:3252.

Thus, self-cleaving RNA molecules such as the Tetrahymena intron and hammerhead ribozyme have been separated into "enzyme" and "substrate" portions that reassociate through Watson-Crick pairing to form an enzyme-substrate complex wherein the active catalytic core assumes an active conformation (Cech, USP 4,987,071; Uhlenbeck, O.C. (1987) *Nature* **328**:596-600; Haseloff, J. & Gerlach, W.L. (1988) *Nature* **334**:585-591). Sequences responsible for substrate binding are generally non-conserved because they do not participate in the catalytic reaction other than by stabilization of the catalytic core in an active configuration.

Designers are essentially free to define a target sequence without alteration of the highly conserved catalytic core. So long as the substrate comprises an appropriate cleavage domain, hybridization of the substrate through Watson-Crick pairing directs the catalytic core into the active conformation (Stage-Zimmerman, T.K., Uhlenbeck, O.C., *RNA*, (1998) **4**:875-889). Such sequences have been described as "substrate recognition helices" (Hertel, K.J., Herschlag, D., Uhlenbeck, O.C., *Biochem* (1994) **33**:3374-3385). Mismatched duplex sequences may form bonds of sufficient strength to stabilize the catalytic RNA in an active conformation and result in cleavage of the non-targeted

sequence. Optimization of complementary sequence length and configuration are examined by Hershlag (*PNAS* 88:6921-25, 1991).

The sequences of catalytic RNA's or ribozymes have evolved to comprise very specific base sequences capable of assuming a catalytic conformation under appropriate conditions. A catalytic core of bases of, for example, the hammerhead ribozyme, has been identified as necessary and sufficient to direct a self-cleaving reaction (Uhlenbeck, O.C. (1987) *Nature* 328:596-600 and Forster, A.C. & Symons, R.H. (1987) *Cell* 50:9-16). The adjacent helices can be varied so long as they permit assembly of the active core structure (Uhlenbeck, O.C. (1987) *Nature* 328:596-600; Haseloff, J., & Gerlach, W.L. (1988) *Nature* 334:585-591; Jeffries, A.C., & Symons R.H. (1989) *Nucleic Acids Res.* 17:1371-1377; Koizumi, M., Iwai, S., & Ohtsuka, E. (1988) *FEBS Lett.* 239:285-288). The sequence requirements for catalytic activity of the core appear to be quite precise. The core of the hammerhead ribozyme for the thirteen conserved nucleotides in seven different natural examples is conserved (Forster, A.C. & Symons, R.H. (1987) *Cell* 49:211-220 and *Cell* 50:9-16; Stage-Zimmerman, T.K., Uhlenbeck, O.C., *RNA*, (1998) 4:875-889; Peracchi, A., Karpeisky, A., Maloney, L., Beigelman, L., Herschlag, D., *Biochem* (1998) 37:14765-14775). Presumably core bases participate in tertiary interactions, coordinate catalytically important divalent metal ions as well as provide functional groups necessary for the activation of the labile bond. Systematic mutagenesis of these bases has confirmed the essential nature of the individual, conserved bases within the core (Ruffner, D.E., Stormo, G.D. & Uhlenbeck, O.C., (1990) *Biochem* 29:10695-10702).

The length of the substrate binding sequences has been investigated. Short sequences enhance catalytic rates by promoting enzyme-product dissociation. Sequences longer than seven have been shown to markedly slow catalytic rate (Herschlag, D., (1991) *PNAS, USA* 88:6921-6925; Hertel, K.J., Herschlag, D. & Uhlenbeck, O.C., (1994) *Biochem* 33:3374-3385; Hertel, K.J., Herschlag, D. & Uhlenbeck, O.C., *EMBO Journal* (1996), 15(14):3751-3757). Specificity is inherently diminished by short sequences but non-specific binding of cleavable substrates is increased with longer sequences. Hertel (1996) notes that the ability of an enzyme to discriminate between two substrates is determined by their relative second order rate constants (K_{cat}/K_m). Wherein the catalysis

proceeds more quickly than a non-specific substrate dissociates, then catalysis of the non-specific substrate occurs. Wherein the rate constants are not sufficiently different between substrate and non-substrate, discrimination is correspondingly diminished. Because substrate binding directs assumption of an active core configuration, non-target sequences are cleaved. Optimization of sequence selection and length enhance catalytic rate and diminish non-specific cleavage events. Tsuchihashi et al. note enhancement of turnover and specificity by the addition of a protein, the p7 nucleocapsid of HIV (Tsuchihashi, Z., Khosla, M., Herschlag, D., *Science* (1993) **262**:99-102).

Regulation of ribozyme activity by a ligand such as RNA or a protein is taught by George, Shih and Bockman (USP 5,834,186). Ribozyme binding to a substrate nucleic acid and assumption of the active conformation of its catalytic core is regulated by interaction of a third molecule. Said molecule need not be RNA but any molecule found by methods taught therein to provide the desired interaction. The same principle is taught by Shih, Bockman and George (USP 5,589,332) wherein the presence of a nucleic acid, protein or other molecule is required for the activity of a ribozyme in the generation of an assayable marker.

The hammerhead ribozyme is exemplary of a catalytic RNA engineered for function in *trans*. The hammerhead ribozyme consists of a highly conserved catalytic core loop and three stems. In nature, these ribozymes act in *cis*. Well characterized examples include the (+)sTRSV (Buzayan, J.M., Gerlach, W.L. & Bruening, G. (1986) *Nature* **323**:349-353; Prody, G.A., Bakos, J.T., Buzayan, J.M., Schneider, I.R. & Bruening, G. (1986) *Science* **231**:1577-1580) ASBV (Hutchins, C.J., Rathjen, P.D., Forster, A.C. & Symons, R.H. (1986) *Nucleic Acids Res.* **20**:3252) and vLTSV (Forster, A.C. & Symons R.H. (1987) *Cell* **49**:211-220). *Trans* activity has been achieved in a variety of ways (Uhlenbeck, O.C. (1987) *Nature* **328**:596-600; Haseloff, J. & Gerlach, W.L. (1988) *Nature* **334**:585-591 & Haseloff (USP 5,494,814); Jeffries, A.C., Symons, R.H. (1989) *Nuc Acids Res* **17**:1371-77; Koizumi, M., Iwai, S., Ohtsuka, E. (1988) *FEBS Lett* **239**:285-88; Koizumi, M., Hayase, Y., Iwai, S., Kamiya, H., Inoue, H. Ohtsuka, *Nuc Acids Res* **17**:17:7059-71) (according to the nomenclature of Hertel, K.J., Pardi, A., Uhlenbeck, O.C., Koizumi, M., Ohtsuka E., Uesugi, S., Cedergren, R., Eckstein, F., Gerlach, W.L., Hodgson, R. & Symons, R.H. (1991) *Nucleic Acids Res.* **20**:3252). The

Haseloff ribozyme permits targeting of a wide variety of target ribonucleic acid sequences which comprise a minimum cleavage sequence by configuring the bases of stems I and III to comprise complements of the sequences flanking the cleavable site. Design parameters including target specificity and substrate turnover must be optimized according to considerations discussed above.

More recently nucleic acid chimeras comprising both DNA as well as RNA have been described which retain or which have augmented catalytic activity (Sawata, S., Shimayama, T., Komizama, M., Kumar, P.K.R., Taira, K., *Nuc Acids Res* (1993) 21(24):5656-5660; Kore, An., Eckstein, F., *Biochem* (1999) 38:10915-10918; Chartrand, P., Harvey, S.C., Ferbeyre, G., Usman, N., Cedergren, *Nuc Acids Res* (1995) 23(20):4092-4096). Catalytic DNA molecules have also been described (Joyce et al., USP 5,807,718).

Exploitation of these newly understood properties of nucleic acids for high sensitivity target nucleic acid detection must overcome background, non-catalytic cleavage. For example, in the detection of rare species, background cleavage events may exceed that generated by specific target-mediated catalytic cleavage. The catalytic rate of the hammerhead ribozyme is 10^6 times faster than background, non-catalytic cleavage (Narlikar, G.J., Herschlag, D., *Annu Rev Biochem* (1997) 66:19-59). However, the number of cleavable molecules subject to non-specific cleavage present in the reaction mixture may be sufficient to generate a background limiting sensitivity. Where the concentration of reaction components is in the micromolar range, a simple calculation based on Avogadro's number suggests that non-catalytic events are likely to exceed catalyst-mediated events.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of a hammerhead ribozyme.

FIG. 2 is another schematic representation of a hammerhead ribozyme.

FIG. 3 is a schematic representation of a hammerhead ribozyme of the invention.

FIG. 4 is a representation of the substrate portion of the hammerhead ribozyme of the invention with a blocking moiety.

FIG. 5 is a representation of a surrogate template of the invention with the blocking moiety catalytically removed.

SUMMARY OF THE INVENTION

The invention is a nucleotide composition for associating with a target nucleic acid; comprising a substrate nucleic acid associated with a core component nucleic acid such that the core component nucleic acid associated with the target nucleic acid forms a catalytic core having biological activity with respect to the substrate nucleic acid. In some embodiments, the substrate nucleic acid and the core component nucleic acid comprise one oligonucleotide. In other embodiments, the core component nucleic acid comprises more than one oligonucleotide sequences.

The invention is also the nucleotide composition associated with the target nucleic acid such that that catalytic core is formed which provides biological activity with respect to the substrate nucleic acid. Preferably, the core component nucleic acid and the substrate nucleic acid are constitutively associated. The association of the nucleotide composition with the target nucleic acid may further create a catalytically active site for the catalytic core. Also preferably, the catalytic core catalytically generates a detectable product. The nucleotide composition associated with the target nucleic acid may further comprises a stabilizing structure such as a flanking nucleic acid helix.

The invention also comprises methods of detecting a target nucleic acid including the steps of providing a nucleotide composition comprising a substrate nucleic acid associated with a core component nucleic acid such that the core component nucleic acid associated with the target nucleic acid forms a catalytic core having biological activity with respect to the substrate nucleic acid; constitutively associating the nucleotide composition with the target nucleic acid; and detecting the formation of the catalytic core.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Unless stated otherwise, the following terms and phrases as used herein are intended to have the following meanings:

The term "sample" or "specimen" refers to nucleic acid isolated from an individual(s) or any nucleic acid containing entity, including but not limited to; skin, plasma, serum, spinal fluid, lymph fluid, synovial fluid, urine, tears, blood cells, organs, tumors, *in vitro* cell culture constituents, bacteria and viruses.

As used herein, the terms "nucleic acid", "polynucleotide" and "oligonucleotide" refer to primers, probes, oligomer fragments to be detected, oligomer controls and unlabeled blocking oligomers and shall be generic to polydeoxyribonucleotides (containing 2-deoxy-D-ribose), to polyribonucleotides (containing D-ribose) and well as chimeric polynucleotides (containing 2-deoxy-D-ribose and D-ribose nucleotides), and to any other type of polynucleotide which is an N glycoside of a purine or pyrimidine base, or modified purine or pyrimidine bases. There is no conceived distinction in length between the term "nucleic acid", "polynucleotide" and "oligonucleotide", and these terms are used interchangeably. Thus, these terms include double-and single stranded DNA, as well as double- and single stranded RNA. The oligonucleotide is composed of a sequence of nucleotides coterminous to a region of the designated nucleotide sequence. A sequence may be of any length so long as the number is greater than one. "Coterminous" means identical to or complementary as defined below to the determined sequence.

The oligonucleotide is not necessarily limited to a physically derived species isolated from any existing or natural sequence but may be generated in any manner, including chemical synthesis, DNA replication, reverse transcription or a combination thereof. The terms "oligonucleotide" or "nucleic acid" refers to a polynucleotide of genomic DNA or RNA, cDNA, semisynthetic, or synthetic origin which, by virtue of its derivation or manipulation: (1) is not affiliated with all or a portion of the polynucleotide with which it is associated in nature; and/or (2) is connected to a polynucleotide other than that which it is connected in nature; and (3) is unnatural (not found in nature). As used herein, the term "PNA" refers to a synthetic oligonucleotide where the linkage between bases is peptide-like unlike the phosphodiester linkage found in nature. The peptide-like backbone is uncharged conferring higher binding force between complementary sequences. They are useful as probes where the target is double-stranded.

Oligonucleotides are composed of reacted mononucleotides to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose

ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage, and is referred to as the "5' end" end of an oligonucleotide if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and subsequently referred to as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. A nucleic acid sequence, even if internalized to a larger oligonucleotide, also may be said to have 5' and 3' ends. Two distinct, non-overlapping oligonucleotides annealed to two different regions of the same linear complementary nucleic acid sequence, so the 3' end of one oligonucleotide points toward the 5' end of the other, will be termed the "upstream" or "forward" oligonucleotide and the latter the "downstream" or "reverse" oligonucleotide. In general, "downstream" refers to a position located in the 3' direction on a single stranded oligonucleotide, or in a double stranded oligonucleotide, refers to a position located in the 3' direction of the reference nucleotide strand.

The term "primer" may refer to more than one oligonucleotide, whether isolated naturally, as in a purified restriction digest, or produced synthetically. The primer must be capable of acting as a point of initiation of synthesis along a complementary strand (DNA or RNA) when placed under reaction conditions in which the primer extension product synthesized is complementary to the nucleic acid strand. Moreover, the amplification product generated by the action of the polymerase incorporates the primer. These reaction conditions include the presence of the four different deoxyribonucleotide triphosphates and a polymerization-inducing agent such as DNA or RNA polymerase or reverse transcriptase. The reaction conditions incorporate the use of a compatible buffer (including components which are cofactors, or which affect pH, ionic strength, hybridization promoting substances such as polyethylene glycol etc. or conditions of the reaction mixture that affect hybridization), at an optimal temperature. In general, this is referred to as the "amplification reaction mixture." In the assays of this invention a promoter sequence serves as the initiation point of polymerase extension along the complementary strand (DNA or RNA). A promoter sequence is not incorporated into the amplification product of the polymerase and may iteratively initiate amplification by the polymerase.

A "complementary nucleic acid sequence" refers to an oligonucleotide which, when aligned with the nucleic acid sequence such that the 5' end of one sequence is paired with the 3' end of the other. This association is termed as "antiparallel." Modified base analogues not commonly found in natural nucleic acids may be incorporated (enzymatically or synthetically) in the nucleic acids including but not limited to primers, probes or extension products of the present invention and may include, for example, inosine and 7-deazaguanine. Moreover, individual nucleic acid sequences may be regarded as complementary wherein they form a stable hybrid duplex with the probe sequence and only with the target sequence when contacted under the conditions of the sample mixture. Implicit within the definition is the discriminatory power imparted by the formation of the hybrid duplex. Where it is desired to differentiate between a target sequence and a non-target sequence comprised by the sample mixture, the formation of a hybrid duplex, under sufficiently stringent conditions, between the target sequence and a probe functionally distinguishes a complementary interaction from a non-complementary one. The complexity of the sample mixture must be considered. While a sequence of four bases is expected to occur randomly once within a sequence of 256 bases, a four base oligonucleotide would not possess sufficient discriminatory power in a mixture comprising the human genome, which comprises three to four billion bases. Complementarity in such a mixture would require the discriminatory power of an oligonucleotide with a target-complementary region of sixteen bases. Thus, a complementary probe, in a sample mixture under stringent conditions, will form a hybrid duplex with the target sequence alone.

As used herein, the term "stringent" is used to describe those conditions of the reaction mixture wherein only a probe comprising a well-matched complementary region will form a hybrid duplex with a target nucleic acid. Moreover, stringency may be functionally defined in terms of those conditions that permit discriminatory binding of the target sequence by a complementary oligonucleotide sequence or probe in a sample mixture. The complexity of the sample mixture must be considered in defining conditions. A sample that comprises a complex mixture of sequences requires more stringent conditions to prevent formation of a hybrid duplex between the probe sequence and a non-target sequence comprised by the sample mixture. Maniatis, T.,

MOLECULAR CLONING; A LABORATORY MANUAL (first and second editions, Cold Spring Harbor Press, Cold Spring Harbor, N.Y.) provides conditions commonly accepted in providing stringent conditions. Conditions provided include both those wherein the probe is contacted with the sample and, second, wherein a wash step is provided to dissociate non-complementary hybrid duplexes. Where it is desired to hybridize under homogeneous conditions, stringency is provided by the conditions under which the probe and target sequence are contacted alone.

The stability of a nucleic acid duplex is measured by the melting or dissociation temperature, or "Tm". The Tm of a particular nucleic acid duplex under specified reaction conditions is the temperature at which half of the base pairs have disassociated.

As used herein, the term "target sequence" or "target nucleic acid sequence" refers to the oligonucleotide that is to be detected in the assays of this invention. The target sequence may be either naturally derived from a sample or specimen or synthetically produced.

As used herein, a "probe" comprises an oligonucleotide that interacts with a region of the target nucleic acid sequence to complete a catalytic core. The probe may comprise the core component nucleic acid alone, or the core component nucleic acid associated with the substrate nucleic acid. The probe may be chimeric, that is, composed in part of DNA, RNA, natural or unnatural bases or comprise linkages unnatural linkages or may comprise non-nucleic acid linker regions. The term, as used herein, is expanded to include nucleic acid complexes that interact through both conventional Watson-Crick base pairing and through non Watson-Crick interactions to form stable complexes so long as said complexes are discriminatory between target and non-target sequences.

As used herein, a "surrogate target sequence", "surrogate sequence" or "surrogate" refers to a sequence generated in the assays of this invention wherein the target nucleic acid is present and, thereby is a surrogate for the presence of the target nucleic acid sequence within the reaction mixture. The surrogate sequence may be directly detected by appropriate means such as gel electrophoresis or indirectly where the surrogate sequence comprises a sequence that specifies an enzyme such as a ribozyme or comprises a promoter sequence for a second polymerase.

The probe may incorporate modified bases or modified linkages to permit greater control of hybridization, polymerization or hydrolyzation. The term "label" refers to any atom or molecule that can be used to provide a detectable signal. Preferably, the signal is detectable in real time and is quantifiable. The detectable label can be attached to a nucleic acid probe or protein. Labels provide signals detectable by either fluorescence, phosphorescence, chemiluminescence, radioactivity, colorimetric (ELISA), X-ray diffraction or absorption, magnetism, enzymatic activity, or a combination of these. The terms "chemiluminescent and bioluminescent" include moieties, which participate in light emitting reactions. Chemiluminescent moieties (catalyst) include peroxidase, bacterial luciferase, firefly luciferase, functionalized iron-porphyrin derivatives and others. The term "absorber/emitter moiety" refers to a compound that is capable of absorbing light energy of one wavelength while simultaneously emitting light energy of another wavelength or as heat energy. This includes phosphorescent and fluorescent moieties. The requirements for choosing absorber/emitter pairs are: (1) they should be easily functionalized and coupled to the probe; (2) the absorber/emitter pairs should in no way impede the hybridization of the functionalized probe to its complementary nucleic acid target sequence; (3) the final emission (fluorescence) should be maximally sufficient and last long enough to be detected and measured by one skilled in the art; and (4) the use of compatible quenchers should allow sufficient nullification of any further emissions.

As used in this application, "real time" refers to detection of the kinetic production of signal, comprising taking a plurality of readings in order to characterize the signal over a period of time. For example, a real time measurement can comprise the determination of the rate of increase of detectable product. Alternatively, a real time measurement may comprise the determination of time required before the target sequence has been amplified to a detectable level. As used in this application, the definition of the word "homogeneous" is expanded to include the configuration of an assay whereby signal amplification reaction and concurrent or subsequent detection of the signal occur in the same reaction mixture.

As used herein, the term "assemble" refers to the close association of nucleic acid sequences such as by but not limited to hybridization. In addition to hybridization, components may be assembled, for example, by covalent bonds. More specifically, the

term refers to sufficiently close affiliation of various regions of oligonucleotides comprised by the amplification reaction mixture that comprise a functioning structure such as a catalytic activity or a catalytic activity-substrate complex.

When used herein the term "primer-dimer" refers to the accumulated short PCR product that is roughly the size of the DNA duplex created by two adjoining primers. These products, however, are not strictly composed of a linear dimer of the two primers. There may be a few template-directed nucleotides between the two primer sequences. While primer-dimers are generated by action of both primers, structures have been described using only a single primer. PCR reactions usually generate some primer-dimer artifacts. ("Target Selection and Optimization of Amplification Reactions" by David H. Persing, p. 96 in "Diagnostic Molecular Microbiology: Principles and Applications", David H. Persing, Thomas F. Smith, Fred C. Tenover, Thomas J. White, American Society for Microbiology, Washington, D.C., 1993.)

As used herein, the term "thermostable" refers to an enzyme, which is relatively stable to heat.

Primer-directed nucleic-acid polymerase activity results in the incorporation of the primer into the amplification product. Thus each copy generated requires the initiation of a hybridization event. A promoter-directed nucleic acid polymerase does not incorporate the promoter sequence. However, a complementary sequence corresponding to a portion of the promoter sequence may be comprised by the amplification product. The promoter, then, once hybridized to a template strand, may act iteratively as the site of initiation of polymerase activity permitting the generation of many complementary copies of the template strand.

The term "conditions of the amplification reaction mixture" refers to assay buffer conditions that allow selective hybridization and enzyme activity optimized for amplification and specificity. The reaction conditions are optimized for co-factors including ionic strength, pH and temperature. These may also comprise other substances such as hybridization enhancing agents, for example, polyethylene glycol.

As used herein the term "hot start" refers to reaction conditions wherein double-stranded nucleic acid sequences are dissociated and primer or probe annealing to the nucleic acid target and extension during amplification is prevented until conditions

including temperature are reached which minimize non-specific events. Further hot start conditions facilitate probe/primer annealing to double-stranded targets.

As used herein, the term "plurality" shall mean two or more of the described entity such as a physical substance or process.

As used herein, the term "multiplex" or "multiplex amplification reaction" refers to the concurrent amplification and detection of a plurality of different target nucleic acid sequences in the same reaction mixture. The term "allele" as used herein refers to genes or nucleic acid sequences that have similar nucleic acid sequences. They may differ by as little as a single nucleic acid base. The term is also used interchangeably with "groups" or "subgroups" where these terms refer to the distinguishable genomes of an organism. The groups or subgroups of Hepatitis C Virus (HCV) are examples.

As used herein, the term "ribozyme" refers to a biologically active molecule comprising a nucleic acid sequence and which interacts with a molecule that comprises an oligonucleotide of which at least one part is RNA, the "substrate", and acts in a sequence-specific manner. A ribozyme comprises a region known as the catalytic core that interacts with the substrate with non-Watson-Crick and non-canonic base interactions. Such interactions provide binding force between the ribozyme and its substrate in addition to other interactions such as Watson-Crick base pairing. These interactions include secondary and tertiary interactions between catalytic core bases, coordination of divalent metal ions as well as interaction of various functional groups. The terms "nuclease activity" and "enzymatic cleavage" refer to a catalytic activity that results in the cleavage of a nucleotide linkage such as at a phosphodiester bond. Ribozymes may possess nuclease activity under specific reaction conditions. In addition, they may possess a variety of other catalytic activities such as phosphorylation, dephosphorylation, ligation, restriction endonucleolysis and polymerization. The catalytic activity is sequence-specific wherein the substrate comprises a specific nucleic acid sequence. It is understood that each of these catalytic activities is within the scope of the biological activities of the ribozymes of this invention. It is understood that ribozymes act catalytically wherein the substrate comprises an appropriate recognition sequence such as an "NUC" or "NUH" sequence (where N is any base, U is uracil or possibly ribo or deoxy-thymidine and C is ribo-cytosine and H is any base other than G) and,

moreover, wherein the conditions of the reaction mixture are appropriate, for example comprise optimum levels of the appropriate divalent metal ions. Where there is no catalytic activity, for example, if the reaction conditions o not permit catalytic activity, such a molecular structure remains a "ribozyme" as used herein. Such a molecule may interact with the substrate as with all or much of the same non-canonic and non-Watson-Crick interactions, and thereby provide utility as a probe.

When used herein, the terms "K_m" and "K_{cat}" are used as conventionally understood in the art. K_m is generally understood to describe hybridization kinetics while K_{cat} is understood to describe the internal catalytic rate of an enzyme.

As used herein, the term "substrate sequence" shall mean a nucleic-acid comprising sequence wherein assembly with the component sequences of the catalytic core results in a conformational change resulting from non-canonic secondary and tertiary interactions between the bases of the substrate sequence and the catalytic core. Wherein such conformational change results in a chemical transition state, said conformational change may result in a catalytic event such as cleavage of the substrate sequence. Wherein such conformational change does not result in a chemical transition state, said conformational state results in a stable interaction of the components of the catalytic core and the substrate sequence mediated by said secondary and tertiary interactions.

As used herein "catalytic core" shall mean those substantially conserved and required bases of a catalytic nucleic acid, which when assembled together with its substrate sequence comprise catalytic or biologic activity. It is understood that catalytic or biologic activity of the catalytic core is dependent upon appropriate alignment of the bases within the catalytic core. Such alignment is dependent upon the bases of the catalytic core, the adjacent duplex stems or other stabilizing structures and conditions of the reaction mixture including divalent metal ions. Said stabilizing structures may include covalent linkages, non-nucleic linkers, avidin-biotin linkages or other linking structures. These may be comprised together with stable stem structure or a stem-structure that is unstable under conditions of the reaction mixture. So long as said stabilizing structure permits stable alignment of the catalytic core components, any structure may serve as an alternative to a stem structure. The catalytic core is distinguished from adjacent regions that aid in said alignment. They may be varied so

long as they provide for the correct alignment of the bases of the catalytic core. Said adjacent regions generally function through Watson-Crick base pairing.

As used herein, the phrases "biologic activity" or "biologically active" shall mean the highly specific interactions that arise from the non-canonic and non-Watson-Crick interactions within the catalytic core and that results in a conformational change within the catalytic core wherein the ribozyme and its specific substrate are assembled. More than one possible conformation is understood. While a conformation that results in an enzymatic transition state may result in a catalytic event, other conformations may be assumed under different conditions that do not result in an enzymatic transition state. Such alternate conformations may provide stable interaction between the components of the complex.

General Method

A biologically active nucleic acid complex is disclosed that comprises a catalytic core, substrate sequence and adjacent nucleic-acid helices wherein said adjacent nucleic-acid helices stabilize the interactions between essential and substantially conserved bases of the catalytic core and target sequence. The bases of the catalytic core are comprised by two or more independent and non-constitutively associated nucleic-acid comprising sequences wherein one of the nucleic acid sequences comprises the diagnostic target. The biologic activity of the catalytic core is dependent upon the association of said sequences of the catalytic core and target sequence.

In a preferred embodiment, the substrate sequence of the biologic activity and one or more of said independent sequences may be constitutively associated wherein at least one of said sequences comprising bases of the catalytic core is not constitutively associated. Said nucleic acid complex may act as a biologically active probe wherein sequence specific interaction of the components are mediated through a combination of Watson-Crick base pairing and non-Watson-Crick, non-canonic interactions mediated through the catalytic core of the complex. Conventionally, such complexes are designated "ribozymes" and described as comprising an enzymatic component and a substrate component comprising the site of enzymatic action. In the following description, this convention will be followed. However, as described above, it is

understood that the conformational changes that arise out of the specific molecular interactions between a ribozyme and its substrate need not be consummated in a catalytic event such as cleavage. Thus it is understood that enzymatic transformation of a substrate molecule is not a necessary property of the molecular complexes of this invention.

Stable secondary and tertiary interactions are generated by the catalytic core of the molecular complexes of this invention only wherein a highly conserved sequence of bases is assembled and appropriate conditions provided. These conserved sequences have evolved naturally and have been characterized, *vide supra*. In addition *in vitro* evolution methods may be employed further defining such conserved sequences by methods exemplified by Ekland and Joyce (Ekland, E.H., Szotak, J.W. & Bartel, D.P. (1995) *Science* 269:364-70; Joyce (USP 6,063,566)).

Such interactions generate a variety of conformational changes. Wherein these changes result in a chemical transition state, enzymatic activity may follow. Other conformational changes that do not provide said transition state also exist (Wedekind, J.E., McKay, D.B., *Annu Rev Biophys Biomol Struct* (1998) 27:475-502); Peracchi, A., Karpeisky, Maloney, L., Beigelman, L., Herschlag, D., *Biochem* (1998) 37:14765-14775). These non-enzymatic conformations are stable only wherein the assembled components together comprise the bases of the catalytic core. Wherein bases within the core are mutated, stable conformational changes do not occur. This property imparts specificity that may be exploited, in preferred embodiments, for the preparation of very specific probes.

Figure 2 is a representation of the catalytic core and substrate sequence of a hammerhead ribozyme, divided to comprise three oligonucleotides, that serves as an example of the nucleic acid complexes of this invention (dashes represent Watson-Crick pairing and dots represent non-Watson-Crick pairing.) The first oligonucleotide is the substrate nucleic acid sequence comprising the catalytic site which is divided along stems I and III from the catalytic core. The catalytic core is divided into two sequences, the first, the core component nucleic acid, comprises the bases and stem regions along stems I and II and the second, the diagnostic target nucleic acid, comprises the bases and stem regions along stems II and III. Wherein the three oligonucleotides are present, the stems

and the bases of the catalytic core are configured to hybridize and assume an active conformation under the conditions of the reaction mixture. Wherein one of the sequences is not present, there is no catalytic or biologic activity. Stem I may be a naturally occurring sequence derived from any naturally occurring hammerhead ribozyme. It may terminate in a stem-loop structure or any non-natural linkage such as through a hydrocarbon linker. Moreover, stem I may be deleted and replaced by any stabilizing structure that permits alignment of the components of the catalytic base wherein other structures such as helices II and III are formed. Bases of helix II of the core component and bases of helix III of the substrate are complementary to the corresponding bases comprised by the target sequence forming helices II and III, respectively. A region of the target nucleic acid is selected that comprises the portion of the catalytic core between helices II and III.

For purposes of illustration, the following conventions will be used to describe the components of the present invention implemented utilizing the hammerhead catalytic core: Stems II and III can be configured to comprise sequences that flank a region of a diagnostically-targeted ribonucleotide, the "diagnostic target", wherein said flanked sequence corresponds to the conserved bases of the catalytic core between stems II and III. An oligonucleotide comprising a catalytic site, the "substrate sequence", is configured to comprise the bases of one strand of stem III. Finally, the sequence comprising the catalytic core between stems I and II, the "core component sequence", is flanked by bases of stem II configured to complement the bases of the diagnostic nucleotide within stem II and to complement the bases of the substrate get within the region corresponding to stem I. The reaction mixture constitutively comprises the substrate and core component sequences. Wherein the diagnostic nucleotide is present, a biologically active complex is assembled. Wherein the diagnostic nucleotide is not present, the catalytic core is incomplete and a biologically active conformation cannot be formed. Thus the molecular complex provides for high sensitivity detection because the catalytic core cannot assemble in the absence of the diagnostic nucleotide.

The molecular complexes of this invention may be comprised entirely of ribonucleic acid. However, they may retain function wherein they are comprised in part by modified bases such as 2' O Methyl RNA bases, unnatural bases or comprise non-

natural linkages. Moreover, they can be comprised in part by deoxyribonucleic acids including the nucleotide 5' to the substrate cleavage site of the hammerhead ribozyme (Chartrand, P., Harvey, S.C., Ferbeyre, G., Usman, N. & Cedergren, R., (1995) *Nucleic Acids Res.* 23(20):4092-96; Kore, A.R. & Eckstein (1999) *Biochem* 38:10915-10918). Methods employing catalytic DNA molecules are taught by Joyce (USP 5,807,718). Unnatural linkers and other covalent linkages may tether components of the ribozyme in addition to complementary base pairing. Moreover, said sequences may be comprised on a single oligonucleotide. Covalently joined, non-nucleotide, moieties may provide additional binding force. An example is provided and methods are taught in USP 5,801,155, which is hereby included in its entirety by reference. Such configurations may provide for constitutive association of components so linked. Triplex nucleic acids may also be utilized in addition or in lieu of the core-flanking helical duplexes. Thus, for example in the hammerhead ribozyme, the substrate sequence and core component sequence may comprise a single oligonucleotide. The regions of the substrate sequence and core component sequence may be linked through a stem-loop structure. It is, however, understood that constitutive association of these components under the reaction conditions may be accomplished by hybridization alone.

Constitutive association of these components may be advantageous. The association of these linked components with the diagnostic nucleic acid sequence eliminates the requirement for multiple, concurrent hybridizations of the components. Wherein multiple hybridizing events are required, simultaneous hybridizations must occur. Such reactions become kinetically disfavored particularly wherein the hybridizing regions are short, the Tm's of the individually hybridizing components are near or lower than the temperature of the reaction mixture or one or more of the components is at low frequency.

The molecular complexes of this invention are necessarily limited to those diagnostic nucleic acids that comprise a suitable core component sequence. However, most larger RNA sequences comprise a sequence suitable for use with one or more of the various known ribozyme catalytic core sequences. At least six different types of ribozymes are known including but not limited to the hammerhead, hairpin, hepatitis delta, *Neurospora* VS, RNAse P and the Intron I and II catalytic sequences. Other RNA

sequences with ligase activity comprise at least seven distinct families (Ekland, E.H., Szotak, J.W. & Bartel, D.P. (1995) *Science* 269:364-70). The ribozymes of this invention may be configured as any of the known ribozymes but is not limited to them wherein others are discovered or produced in the future. Certain limitations are imposed by the presence of a purine 5' to a GAAA sequence forming the conserved sequence between stems II and III of the hammerhead ribozyme. The first base of stem II optimally requires a pyrimidine in this position (Tuschl, T. & Eckstein, F. (1993) *PNAS, USA* 90:6991-6994; Ruffner, D.E., Stormo, G.D. & Uhlenbeck, O.C. (1990) *Biochem* 29:10695-10702). However, a closely related hammerhead ribozyme, the satellite RNA of the barley yellow dwarf virus (sBYDV), comprises two additional bases in the conserved catalytic core and utilizes a purine in the conserved position 5' to the GAAA sequence and a slightly altered sequence between stems I and II comprising CUGAUGA (Nakamaye, K.L. & Eckstein, F. (1994) *Biochem* 33:1271-1277). In the mutagenesis studies of Ruffner et al., certain base substitutions in stem-closing bases of stems II and III are tolerated. Bases within the adjacent Watson-Crick pairing helices act only to stabilize components (Hertel, K.J., Peracchi, A., Uhlenbeck, O.C., Herschlag, D., *PNAS, USA* (1997) 94(16):8497-502).

Herschlag provides a thermodynamic analysis of the optimum length of complementary sequence regions of ribozymes (Herschlag, D., (1991) *PNAS, USA* 88:6921-6925) and observes that longer sequences do not necessarily provide greater specificity. The individual regions of the molecular complexes of this invention hybridizing with the diagnostic nucleic acid sequence are thus preferably kept short. The individual hybridizing regions of constitutively associated components may not individually hybridize with the diagnostic sequence under the reaction conditions but together provide a sufficient annealing force to form a stable hybrid bond.

Narlikar et al. suggest that in the absence of sufficient binding energy, such as through Watson-Crick base pairing, to ensure stable docking of a ribozyme and its substrate sequence in the ground state, additional interactions are provided through functional groups within the catalytic core wherein the ribozyme assumes a closed conformation (Narlikar, G.J., Herschlag, D. *Biochem* (1998) 37(28):9902-11). Likewise, the diagnostic nucleotide, comprising bases within the catalytic core, can be thus

expected to enjoy enhanced binding mediated through such interactions. The annealing forces localized within the catalytic core may permit shortening of sequences participating in the non-core, Watson-Crick pairing regions of adjacent nucleotides. Advantages of enhanced specificity can be expected.

In addition, catalytic core bases provide functional specificity in that bases within this region are essential for post-binding catalytic activity. In the absence of the core component sequence, no ribozyme can be constituted and background catalytic activity resulting from the misdirected activity of conventional ribozymes is completely eliminated.

Crystallographic studies have demonstrated marked conformational changes upon assembly of the specific components of the hammerhead ribozyme. However, many of the studies have not demonstrated positioning of the active site in a conformation that allows cleavage (Wedekind, J.E., McKay, D.B., *Annu Rev Biophys Biomol Struct* (1998) 27:475-502). These "non-catalytic" conformations may be relevant to the overall reaction pathway forming an intermediary prior to the highly specific interactions culminating in a catalytic event. Nevertheless, the intermediate so discerned comprises the essential non-canonic interactions of the active catalytic core. Rearrangements occurring within the ribozyme upon assuming such closed forms increase the binding force. Moreover, interactions involving non-target sequences involving conserved bases within the catalytic core are correspondingly disfavored because said rearrangements do not occur. Wherein the molecular complexes of this invention are utilized as catalytic or biologically active probes, they provide advantages of specificity over conventionally hybridizing probes. The representation of the hammerhead ribozyme structure, as solved by Pley, H.W., Flaherty, K.M., McKay, D.B. (1994), *Nature* 372:58-74 (dashes represent Watson-Crick pairing and dots represent non-Watson-Crick pairing), shown in Fig. 3 exemplifies the potential specificity. The bases G-A-A of the catalytic core between stems II and III "stack un-interrupted with those of Stems II and III, leading to the near-collinearity of the stems." (p 489, Wedekind, J.E., McKay, D.B., *Annu Rev Biophys Biomol Struct* (1998) 27:475-502) A schematic stereo representation in the same article displays the sequence corresponding to the target strand forming a continuous helix comprising stems II and III.

A variety of means favor the formation of the non-catalytic rearrangements over those that lead to a catalytic event. As exemplified by the hammerhead ribozyme, catalytic activity may depend upon the presence of a 2' hydroxyl group at the base 5' of the cleavage site, (Chartrand, P., Harvey, S.C., Fereyre, G., Usman, N., Cedergren, R., *Nuc Acids Res* (1995) 23(20):4092-4096). Mutation of this base into a base that does not comprise said 2'hydroxyl or some other base or condition within the reaction mixture that does not permit catalysis, generates a stable complex. Thus a structure or complex that interacts with the diagnostic nucleotide to form a non-catalytically active core is configured to act as a probe for the diagnostic nucleotide. Wherein a mismatch within or adjacent to the catalytic core destabilizes interactions therein and thereby prevent stable association of the components, the probe can be expected to provide higher specificity than a conventional probe.

The probes of this invention provide advantages according to the optimization of Herschlag. Specific, uninterrupted interaction is formed along the complementary regions of the catalytic substrate and ribozyme component through the intervening thermodynamically stabilizing interactions provided by the non-Watson-Crick, non-canonic interactions within the catalytic core region. The canonic, Watson-Crick interactions need not hybridize stringently themselves for a highly specific interaction to occur with the probes of this invention. Wherein the complementary regions are five bases each and the catalytic region comprises three additional bases, the interaction comprises thirteen bases. Wherein complementary regions comprise nine bases each, the interaction comprises twenty-one uninterrupted bases. Moreover, specificity is comprised in the total number of all the interacting bases.

In diagnostic applications, the molecular complexes of this invention find utility as catalytic probes where their innate biologic activity provide significant advantages over conventional probes. Hogan, USP 5,424,413, generates a cleaved probe as a surrogate for the presence of a target sequence. A restriction-endonuclease substrate is created by the hybridization of two independent probes wherein the probe regions comprising the components of the restriction site are too short to hybridize independently of concurrent hybridization of target-complementary regions of each of the probes that brings them into juxtaposition. The specificity of such reactions is limited by the

interaction of the enzyme. Wetmur divides hybridization into two discrete events, nucleation and "zippering" (Wetmur, J.G. "DNA probes: applications of the principles of nucleic acid hybridization", Crit Rev Biochem Mol Biol. (1991), 26(3-4):227-59). The first step is relatively temperature independent and involves three to four bases and would occur at temperatures above the predicted Tm of the hybridizing regions. A nucleation event creating a short restriction site would be recognized as a substrate for the appropriate restriction endonuclease comprised by the reaction mixture. The association of said restriction site and enzyme results in a high local concentration of the complementary bases resulting in their stable association much like the high local concentration of the complementary bases of a hairpin structure, potentially in the absence of the target nucleic acid sequence. The catalytic probes of this invention overcome the difficulties of such strategies because they are not subject to such interactions.

In preferred embodiments, the molecular complexes of this invention are first utilized as a probe and subsequently as a ribozyme. Stable structures can be produced wherein catalytic conditions within the reaction mixture are not present, for example, inadequate divalent metal ion concentrations. Thus catalytic activity can be delayed until appropriate conditions for catalysis are established. A complex configured to act as a probe and the diagnostic nucleotide may be permitted to form a stable complex and then separated from a reaction mixture comprising non-reacted components. Conditions of the reaction mixture comprising the isolated complex can then be altered to permit catalytic action. This embodiment overcomes background events that arise from non-catalytic cleavage of components within the sample matrix. Thus the adaptable nature of the molecular complexes of this invention is of significant advantage for specific detection of rare sequences comprised within complex mixtures.

A catalytic substrate may be configured so that one end of the molecule comprises a blocking moiety as shown in Fig. 4. A portion of a surrogate template is configured at the other end. Said surrogate template portion comprises a probe binding site and a first primer site. It is understood that the blocking moiety can be comprised at the other end. (For purposes of this illustration, the substrate is comprised of DNA bases save for a single RNA base 5' to the cleavage site.) Wherein conditions are provided within the

reaction mixture to permit catalytic cleavage, the portion of the surrogate template is catalytically separated from the blocking moiety. As shown schematically in Fig. 5, the surrogate template for a polymerase chain reaction has been catalytically separated from the blocking moiety. Following correction of the 3' end to comprise a 3' OH group, an oligonucleotide comprising a first region complementary to the cleaved end is permitted to hybridize under suitable conditions. A second portion of said oligonucleotide comprises a nucleic acid sequence complementary to a region comprising a second primer site and subjected to the action of an appropriate nucleic acid polymerase and reaction mixture comprising appropriate nucleotides wherein the reaction generates a full length surrogate template. The surrogate template may be detected in the polymerase chain reaction. (N is any base; N' is the complement of N, H is any base but G and H' is the complement of H. (Dashes represent Watson-Crick pairing.) Upon cleavage, the terminal base may require enzymatic change such as the transfer of a phosphate group to a terminal 5' hydroxyl group to make it competent for ligation. An example is T_4 polynucleotide kinase (available with instructions from Promega Corporation). Likewise, the 3' end can be treated with alkaline phosphatase to remove a phosphate at the 3' end (available with instructions from Promega Corporation). Two examples are provided wherein an amplifiable and detectable surrogate template is produced wherein the blocking moiety is thus removed.

In the first example, a first oligodeoxynucleotide may be provided that preferably incorporates uracil bases. It comprises a region complementary to the cleaved end of the catalytic substrate. A second oligonucleotide may be provided that is complementary to said first oligonucleotide wherein a nicked duplex between the catalytic substrate and said second oligonucleotide is formed under the conditions of the reaction mixture. Said duplex can be treated with an appropriate nucleic acid ligase such as T_4 ligase to form a contiguous oligonucleotide (available with instructions from Promega Corporation). Said first oligodeoxynucleotide comprising uracil bases may be treated with uracil N glycosylase (available with instructions from Life Technologies Inc.) to destroy the oligonucleotide. The catalytic substrate is so configured that it is not amplifiable, but upon ligation to said second oligonucleotide is amplifiable. The catalytic substrate may comprise a first primer site and probe site. Said second oligonucleotide may comprise a

second primer site. The ligated sequence is competent for exponential amplification and detection by such strategies, for example, as PCR or TMA. Other methods are conceivable and may employ RNA replicases.

In the second example, the 3' end of the cleaved probe is extended by a suitable DNA polymerase such as T_4 DNA polymerase (available with appropriate instructions from Promega Corporation). An oligodeoxynucleotide, preferably comprising uracil bases that forms a duplex under conditions of the reaction mixture comprises an overhanging free end that acts as a template for the polymerase. Subsequently, the complementary oligodeoxynucleotide may be treated with uracil N glycosylase to destroy the oligonucleotide. In a similar manner to the example above, a surrogate template is created for subsequent amplification and detection.

Separation of the molecular complex comprising the diagnostic sequence can be accomplished in a number of ways. In a preferred embodiment, the diagnostic nucleotide may comprise a region complementary to an oligonucleotide that may be tethered to a support that can be added facilitating washing steps. The Tm of this interaction may be configured to be below an amplification or detection temperature such as encountered in the polymerase chain reaction, thereby freeing the components into the liquid phase.

Wherein said processes result in the assembly of a surrogate target competent for amplification and detection by the reaction components of the system, such methods are within the scope of this invention. Moreover, the ligases of Ekland are within the scope of this invention and may be configured as molecular complexes of this invention. The ligation of the two catalytic substrates can provide a surrogate target sequence directly and can be used in similar strategies as described above.

The surrogate sequence or its amplification product can be detected by a number of different techniques. The product may be detected by the interaction of the nucleic acid with a nucleic acid binding agent such as ethidium bromide. Said detectable signal may be detected and quantified in a variety of devices such as a fluorometer such as the Perkin-Elmer LS50B or by gel electrophoresis. It can also be detected in the same reaction mixture with a nucleic acid binding agent that result in a change in a detectable signal upon binding. Examples are intercalating agents such as ethidium bromide. A nucleic acid binding agent that undergoes such changes upon binding RNA and results in

a change in a detectable signal can be used where the polymerase used is a DNA- or RNA-dependent RNA polymerase. A variety of such agents are available from Molecular Probes, Inc.

Labeled-probe techniques permit integration of detection and amplification into a single reaction mixture permitting homogeneous amplification and detection. Livak et al., *PCR Methods and Applications*, 4:357(1995), offers an example of this strategy by teaching the use of a signal fluorescent dye and a quencher fluorescent dye attached to the 5' and 3' ends of an oligonucleotide probe. Thus, the presence of the target nucleic sequence may be measured by detecting fluorescence of the signal fluorophore generated by nucleolysis of the labeled strand provided in the amplification reaction mixture.

Fluorescence generated upon cleavage of a labeled-RNA probe configured with a signal and quencher fluorophore can be directly monitored. The use of labeled RNA probes permits multiplex analysis within a single reaction mixture. A multiplicity of different labeled-RNA probes may be included in the same reaction mixture permitting their sequence-specific cleavage by concurrently generated ribozymes. By labeling the RNA probes with spectrally distinct fluorophores, individual amplification reactions can be monitored within the same reaction mixture. In a preferred embodiment, at least one of the reactions can be used as an internal control or standard. Further, alleles may be discriminated by the use of a multiplicity of allele-specific probes within the same reaction mixture.

The ribozyme-mediated catalytic event can also be coupled to redox, hydrolysis or other reactions that can terminate in the modification, such as by oxidation, reduction or hydrolysis of a detectable signal-generating substance such as a chemiluminescent substrate or a color-generating compound. Where the product of enzymatic action is rendered insoluble or otherwise immobilized, the system becomes useful in applications involving in-situ hybridization.

Other catalytic activities of ribozymes may be exploited for the generation of a detectable signal. For example, polymerase activity of the ribozyme generated in the reaction may be monitored by detecting a change in electrophoretic mobility of a labeled ribozyme-target sequence. Likewise, phosphorlyase activity of the ribozyme generated in the reaction may be monitored by a shift in electrophoretic activity based on a change in

mass to charge ratio of the labeled-ribozyme target sequence. Methods are taught in USP 5,093,246, which is hereby incorporated by reference.

In a preferred embodiment, the amplified-surrogate sequence may be detected in a sequence-specific manner by a Molecular Beacon™. Tyagi and Kramer (*Nat Biotechnol*;14(3):303-8 (1996 Mar)) describe labeled-nucleic acid probes that recognize and report the presence of specific nucleic acids in homogeneous solutions. Sequence complementarity at the 5' and 3' ends of the nucleic acids results in self-annealing in the absence of the target nucleic acid sequence while sequence complementarity to the target nucleic-acid sequence favors annealing of the nucleic acid probe to the target nucleic-acid sequence resulting in a fluorogenic conformational change in the probe. The probe is particularly suited to homogeneous applications such as real time amplification monitoring in a sealed tube. In this embodiment, ribozyme-mediated cleavage of a labeled-sequence specific probe is not required. The probes may be DNA as well as RNA.

A number of detectable labels which would be suitable for use in this invention, as well as methods for their incorporation into the probe, are known in the art and include, but are not limited to, enzymes (e.g., alkaline phosphatase and horseradish peroxidase) and enzyme substrates, radioactive atoms, fluorescent dyes, chromophores, chemiluminescent/bioluminescent labels, electrochemiluminescent labels, labeled receptor-ligand binding, labeled antibody-antigen coupling, or any other labels that may interact with each other to enhance, alter, or diminish a detectable signal in real time. Should elevated temperatures be achieved during the course of an assay, the label must be able to survive the high temperature.

In a preferred embodiment, the interactive labels comprise a signal (such as a fluorescein) and quencher (such as rhodamine) fluorescent dye pair. Two modes of fluorescence quenching are available with the reporter substrates of this invention. The first utilize fluorescence resonance energy transfer described in USP 5,853,990. The fluorescence may be measured in any suitable way, including the LS-50B System (Perkin-Elmer) or Model 7700 Sequence Detector (Perkin-Elmer). A second method of quenching is available whereby the energy of the first fluorophore is prevented from fluorescing by a direct interaction with the quenching moiety when in very close

proximity or is dissipated as heat by the presence a non-fluorescing quenching moiety as in a dark quencher. (Tyagi and Kramer, Nat Biotechnol,14(3):303-8 (1996). A preferred quenching moiety used in these applications is DABCYL available from Glen Research, Inc. Such a quenching moiety can be attached in close proximity to a quenching fluorophore of a resonance energy transfer pair. Prior to interaction of the reporter substrate with the reporter, the signal fluorophore transfers its energy to the quenching fluorophore, which, in turn, is quenched or otherwise prevented from fluorescing by the quenching moiety.

A number of modifications may be made to the probe to maximize quenching prior to hybridization and release. In general, the dyes may be attached either at the termini or sub terminally. Alternatively, the dyes may be attached internally to optimize detection characteristics. Or, the probe can be designed so that it forms a secondary structure, such as a hairpin, that brings the signal and quencher into proximity prior to interaction with the signal. The use of ribo-oligonucleotides or unnatural analogues such as 2' O methyl RNA oligonucleotides may be used to great advantage in this embodiment. RNA forms inherently more stable secondary structures than DNA or chimeric oligonucleotides. Accordingly, probes can be designed which very efficiently quench signal fluorescence prior to hybridization leading to assay systems with very low background noise.

In a similar embodiment, detection of the cleaved, labeled reporter target can be achieved using fluorescence polarization. This technique is able to distinguish between large and small molecules based on molecular tumbling. Large molecules (e.g., intact labeled probe) tumble in solution much more slowly than small molecules. Upon linkage of a fluorescent moiety to the molecule of interest, this fluorescent moiety can be measured (and differentiated) based on molecular tumbling, thus differentiating between intact and digested probe. Detection may be measured in conjunction with PCR using LS-50B fluorimeter (Perkin Elmer) or other device configured to detect a fluorescence polarization signal.

In yet other embodiments, the use of radioactive atoms, such as ^{32}P, may be suitable for labeling and detection. Enzymatic methods for introducing ^{32}P into nucleic acids are known in the art, and include, for example, 5' end labeling with polynucleotide kinase, or random insertion by nick translation and the Klenow fragment. Labels at the 3' terminus

may employ polynucleotide terminal transferase to add the desired moiety, such as for example, cordycepin ^{35}S-dATP, and biotinylated dUTP. The labels may be attached to the ribo- or chimeric oligonucleotide probe directly or indirectly by a variety of techniques. Depending on the precise type of label used, the label might be located at the 5' or 3' end of the probe, located internally in the probe's nucleotide sequence, or attached to carbon spacer arms of various sizes and compositions to facilitate signal interactions. Using commercially available phosphoramidite reagents, one can produce oligomers containing functional groups (e.g., thiols or primary amines) at either terminus via an appropriately protected phosphoramidite. Enzymes can be detected by their activity on a secondary substrate.

Methods for introducing oligonucleotide functionalizing reagents to introduce one or more sulfhydryl, amino or hydroxyl moieties into the oligonucleotide probe sequence, typically at the 5' terminus are described in USP 4,914,210. A 5' phosphate group can be introduced as a radioisotope by using polynucleotide kinase and γ-^{32}P-ATP to provide a signal group. Biotin can be added to the 5' end by reacting an aminothymidine residue, introduced during synthesis, with an N-hydroxysuccinimide ester of biotin.

Oligonucleotide (DNA and RNA) derivatives are also available labels. For example, etheno-dA and etheno-A are known fluorescent adenine nucleotides, which can be incorporated into a ribo-or chimeric oligonucleotide probe. Similarly, etheno-dC is another analog that could be used in probe synthesis. The probes containing such nucleotide derivatives may be hydrolyzed to release much more strongly fluorescent mononucleotides upon cleavage.

The oligonucleotide and labeled oligonucleotides may be prepared by a number of methods. Methods for preparing oligonucleotides (deoxy-, ribo-, and chimeric) of a specific sequence are known in the art, and include, for example, cloning and restriction of appropriate sequences, direct automated chemical syntheses and enzymatically. Such techniques include, for example, the phosphotriester method, that phosphodiester method, the diethylphosphoramidate method, and the solid support method.

The composition of the oligonucleotides can be designed to inhibit nuclease activity. The incorporation of modified phosphodiester linkages (e.g., methyl phosphorylthioate or

methylphosphonates) in the labeled oligonucleotides during chemical synthesis may be used to prevent cleavage at a selected site.

In the practice of this invention, it may be desirable to control the melting temperature of the probes. The invention allows for significant optimization of this characteristic as opposed to the prior art systems limited to DNA oligonucleotide probes. Hybrids comprising natural and unnatural bases and linkages may have a higher melting temperature than DNA:DNA or chimeric:DNA hybrids of the same base composition permitting greater specificity and control over the Tm. The length of complementary nucleic acids is also known to affect the hybridization rate and the relative stability of the duplexes. Shorter nucleic acid molecules generally require a cooler temperature to form sufficiently stable hybrid complexes with the target nucleic acid. Therefore, the target sequence complementary region can be designed optimized to hybridize with maximum efficiency and specificity. One can also vary the base composition of the probes to affect thermal stability. For example, the nucleotide composition of the probes can be chosen to have greater G/C content.

Multiplexing, especially where it is desired to detect or quantify a number of different nucleic acid targets in a single reaction mixture. Primer-directed methods are particularly subject to the production of non-target sequence amplification products including primer-dimers. The assays of this invention are not subject to the generation of these unwanted amplification products.

Nucleic acid sequences can be synthesized in a nucleic acid synthesizer as described below using available phosphoramidites that are functionalized to comprise a group that can be chemically linked. One can select from such phosphoramidites available from Glen Research. Functionalized clear plastic surfaces are available on microtiter plates selected from those available from Nunc. Cross-linking reagents and instructions are available from Pierce Chemicals.

Template-dependent copying of the oligonucleotide from a site of initiation specified by a promoter is catalyzed by a polymerizing agent in the presence of adequate amounts of the four deoxyribonucleoside triphosphates (dATP, dGTP, dCTP, and dTTP) or analogs discussed above, in a reaction medium which is comprised of the appropriate salts, metal cations and pH buffering system. Suitable polymerizing agents are enzymes

known to catalyze promoter initiated and template-independent and template-dependent DNA or RNA synthesis. Known and unknown polymerases suitable for the practice of this invention include, for example, SP6, T3 and T7 DNA dependent RNA polymerases ("Molecular Cloning, A Laboratory Manual", 2nd edition (1989), J. Sambrook, E.F. Fitsch, T. Maniatis, Cold Springs Harbor Press). The reaction conditions for catalyzing DNA or RNA synthesis with these polymerases are well known in the art.

The products of the synthesis are duplex molecules consisting of the template strands and the primer extension strands, which include the target sequence. By-products of this synthesis are labeled oligonucleotide fragments, which consist of a mixture of mono-, di-, and larger nucleotide fragments.

In a preferred method, the assays of this invention are carried out as an automated process. Any thermally controlled device is suitable. However, a DNA thermal cycler, such as the commercially available machine from Perkin-Elmer/ABI Instruments may be employed because they permit convenient modulation of the temperature that may be used in the course of certain embodiments. The use of a machine such as the Perkin-Elmer Model 7700 Sequence Detector is particularly useful in certain embodiments because spectal data can be collected concurrent with the signal generation process facilitating real time detection.

The oligonucleotide and labeled probes may be prepared by a number of methods. Methods for preparing oligonucleotides (deoxy-, ribo-, and chimeric) of a specific sequence are known in the art, and include, for example, cloning and restriction of appropriate sequences, direct automated chemical syntheses and enzymatically. Such techniques include, for example, the phosphotriester method, the phosphodiester method, the diethylphosphoramidate method, and the solid support method.

Detection of the oligonucleotides generated during the assays may be accomplished by a variety of methods. One convenient embodiment of the invention is to subject the reaction products to size analysis. Methods for determining the size of the labeled nucleic acid fragments are known in the art, and include, for example, gel electrophoresis, sedimentation in gradients, gel exclusion chromatography and homochromatography.

Reagents employed in the methods of the invention can be packaged into kits and diagnostic kits. Kits and diagnostic kits include the labeled oligonucleotides and the

probes/primers together or in separate containers. If the oligonucleotide is unlabeled, the specific labeling reagents may also be included in the kit. The kit may also contain other suitably packaged reagents and materials needed for amplification, for example, buffers, dNTPs, and/or polymerizing means, and for detection analysis, for example, enzymes and solid phase extractants, as well as instructions for conducting the assay.

EXAMPLE

Sequences (RNA):

1. Substrate: 5' GCU UCC AUG UCG GCA GAA UGC (SEQ ID NO. 1)
2. Core component: 5' AUU CUG CCC UGA UGA GUC CGC C (SEQ ID NO. 2)
3. Target sequence: 5' GGC GGA CGA AAC AUG GAA (SEQ ID NO. 3)
4. Target sequence (mutanized): 5' GGC GGA CGA CAC AUG GAA (SEQ ID NO. 4)

RNA sequences are prepared in the following manner: RNA is synthesized utilizing a ABI 392 synthesizer using RNA phosphoramidites and liquid reagents, as specified by PE-Biosystems, provided by Glen Research. RNA is phosphate and silyl-deprotected using protocols provided by Glen Research. RNA is reverse phase HPLC purified on a PE Series 200 HPLC using a 10-30 percent gradient of acetonitrile in 0.1 M TEAA. Appropriate fractions are collected, desalted and dried in a Savant SpinVac. The 5' trityl protecting group is removed using protocols provided by Glen Research. Quantification is performed on a Beckman DU 650 configured with nucleic acid analysis software. The RNA is 5' end-labeled with γ ^{32}P ATP (New England Nuclear) following T4 polynucleotide kinase exchange reaction protocol of Life Technologies and enzyme provided by Life Technologies.

Cleavage reactions contain .2μM substrate, .3μM of each (ribozyme component and target sequence), 50 mM Tris-HCl (pH 8) and 10mM $MgCl_2$. The enzyme components are combined in the presence of buffer and incubated at 60°C for one minute. The reactions are started by adding $MgCl_2$, to a final concentration of 10mM at 37°C. The reactions are stopped by the addition of one volume of 50mM EDTA. To demonstrate the action of the composition as a probe (where no cleavage is desired), $MgCl_2$ is left out.

Electrophoresis of the respective components is performed as follows: A 15 per cent polyacrylamide TBE-urea gel from BioRad, is used following their protocol. Gels are transferred to a Ambis 4000 radionuclide scanner for imaging. Appropriate size markers are end labeled as above and run concurrently.

Exemplary results:

Lanes:

1. Molecular weight markers
2. Substrate alone at 6768
3. Cleavage reaction (run with $MgCl_2$) one band: 3483 (second fragment not labeled)
4. Reaction run without $MgCl_2$: band at 6768
5. Mutant run (run with $MgCl_2$): band at 6768

All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

While the present invention is described herein in terms of certain preferred embodiments, those skilled in the art will recognize that various modifications and improvements may be made to the invention without departing from the scope thereof. Moreover, although individual features of one embodiment of the invention may be discussed herein or shown in the drawings of the one embodiment and not in other embodiments, it should be apparent that individual features of one embodiment may be combined with one or more features of another embodiment or features from a plurality of embodiments.

WHAT IS CLAIMED IS:

1. A nucleotide composition for associating with a target nucleic acid; comprising a substrate nucleic acid associated with a core component nucleic acid such that the core component nucleic acid associated with the target nucleic acid forms a catalytic core having biological activity with respect to the substrate nucleic acid.

2. The nucleotide composition of claim 1, wherein the substrate nucleic acid and the core component nucleic acid comprise one oligonucleotide.

3. The nucleotide composition of claim 1, further comprising the target nucleic acid.

4. The nucleotide composition of claim 1, wherein the core component nucleic acid comprises more than one oligonucleotide sequence.

5. The nucleotide composition of claim 1, wherein the catalytic core has a catalytic activity selected from the group consisting of restriction endonucleolysis, ligation, phosphorylation, dephosphorylation, and polymerization.

6. The nucleotide composition of claim 3, wherein the composition is chimeric comprising non-ribonucleotide bases or non-nucleic portions.

7. The nucleotide composition of claim 3, wherein the substrate nucleic acid and the core component nucleic acid and the target nucleic acid are constitutively associated.

8. The nucleotide composition of claim 7, wherein the constitutive association comprises hybridization.

9. The nucleotide composition of claim 7, wherein the constitutive association comprises covalent linkage.

10. The nucleotide composition of claim 7, wherein the constitutive association comprises hybridization and covalent linkage.

11. The nucleotide composition of claim 7, wherein the constitutive association creates a catalytically active site for the catalytic core.

12. The nucleotide composition of claim 3, wherein the nucleotide composition is configured so that the catalytic core catalytically generates a detectable product.

13. The nucleotide composition of claim 3, wherein the nucleotide composition associated with the target nucleic acid further comprises a stabilizing structure.

14. The nucleotide composition of claim 13, wherein the stabilizing structure comprises a flanking nucleic acid helix that stabilizes the catalytic core.

15. The nucleotide composition of claim 1, wherein the core component nucleic acid comprises a sequence selected from the group consisting of C-G-A-A-A and G-A-A-A.

16. The nucleotide composition of claim 15, wherein the target nucleic acid comprises a sequence selected from the group consisting of N-C-U-G-A-N-G-A and N-C-U-A-C-G-A-C, wherein N is any ribonucleotide.

17. The nucleotide composition of claim 16, wherein the substrate nucleic acid comprises the sequence N-U*-H, wherein N is any ribonucleotide and U* is uracil or deoxythymidine and H is any ribonucleotide except G.

18. The nucleotide composition of claim 3, wherein the substrate nucleic acid further comprises a first hybridizing sequence and wherein the target nucleic acid has a region complementary to the first hybridizing sequence.

19. A method for detecting a target nucleic acid, comprising the steps of:

a) providing a nucleotide composition comprising a substrate nucleic acid constitutively associated with a core component nucleic acid such that the core component nucleic acid associated with the target nucleic acid forms a catalytic core having biological activity with respect to the substrate nucleic acid;

b) associating the nucleotide composition with the target nucleic acid; and

c) detecting the formation of the catalytic core.

20. A nucleotide composition for associating with a target nucleic acid, wherein the composition associated with the target nucleic acid comprises a ribozyme having a catalytic core and first and second flanking nucleic-acid helices; comprising a substrate nucleic acid comprising first and second hybridizing sequences associated with a core component nucleic acid comprising a hybridizing sequence complementary to the first hybridizing sequence of the substrate oligonucleotide and a portion of the catalytic core.

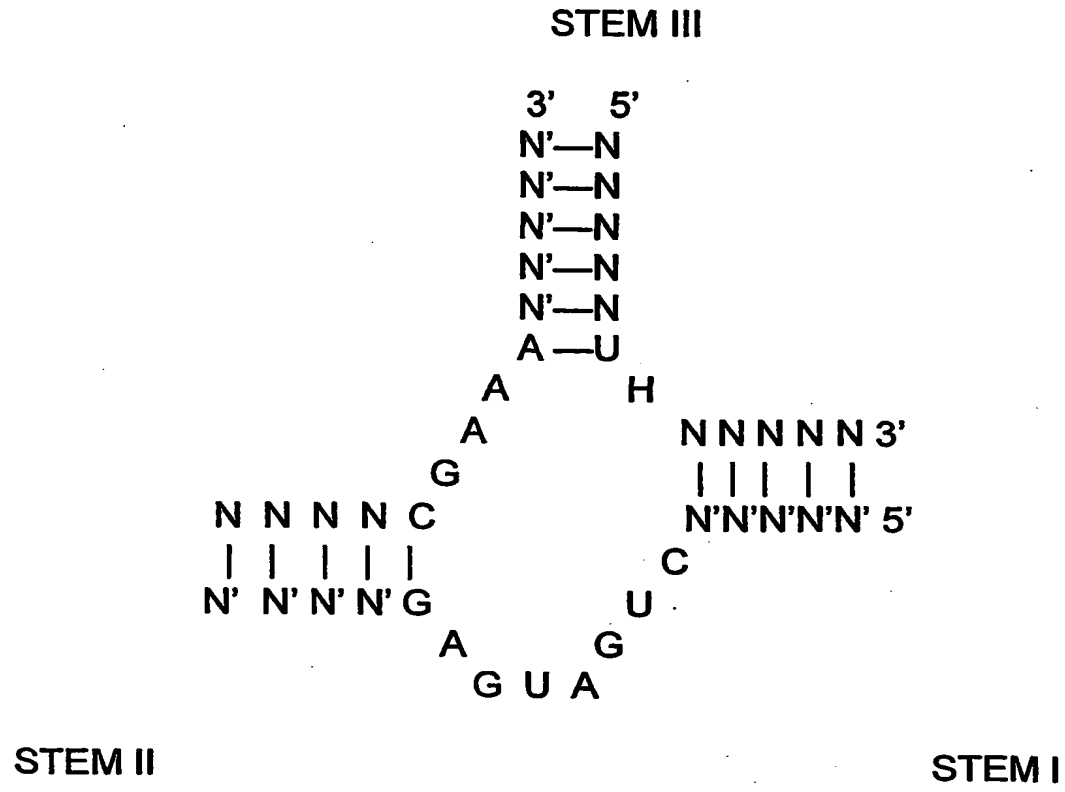

FIG. 1

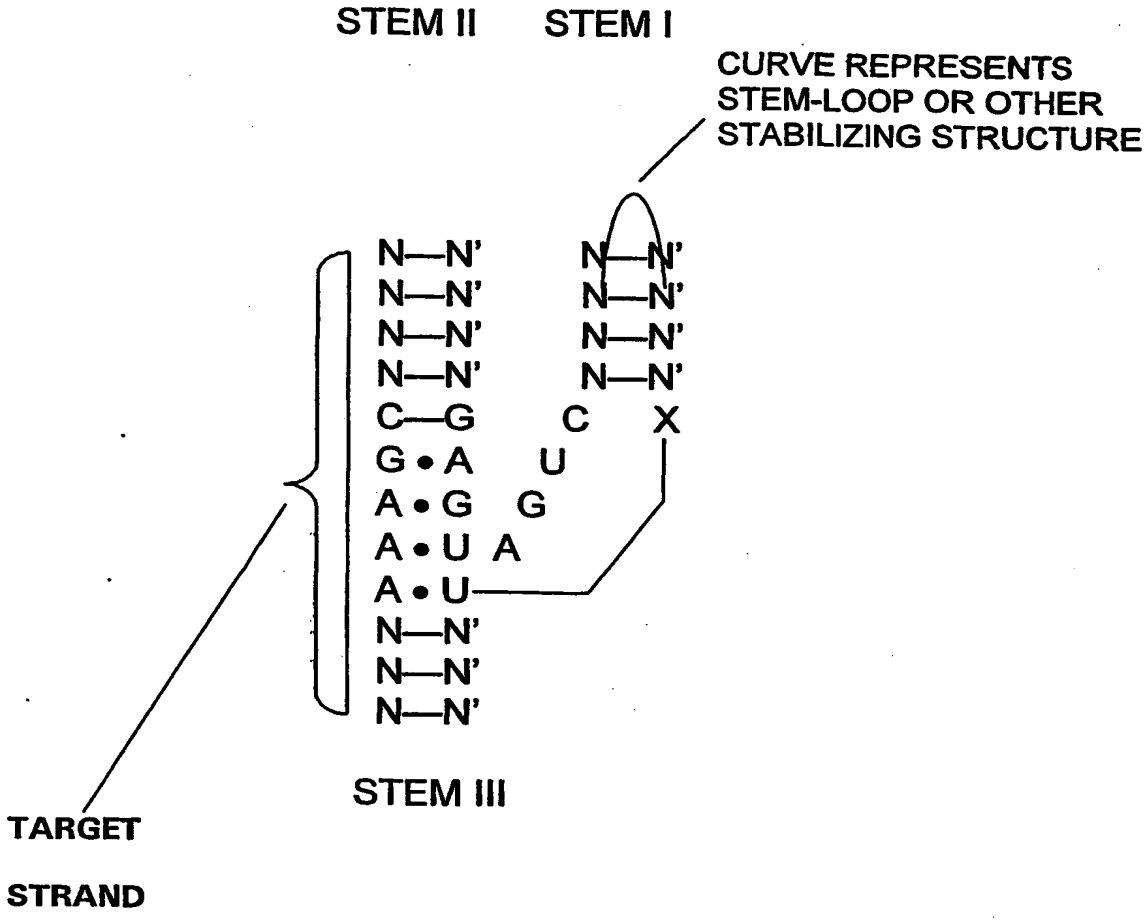

FIG. 2

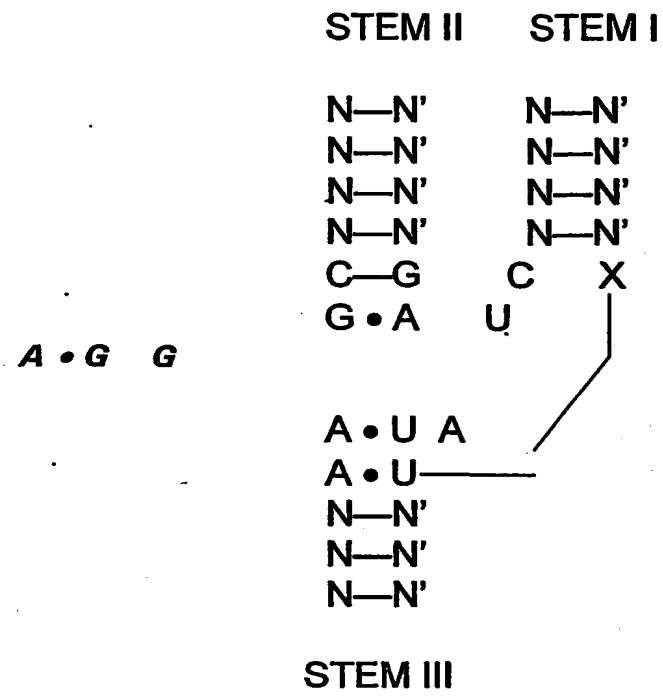

FIG. 3

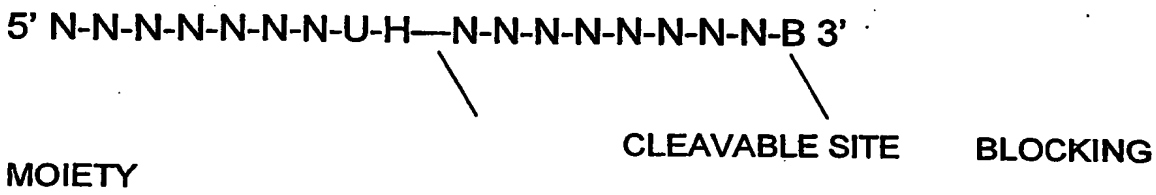
5' N-N-N-N-N-N-N-U-H—N-N-N-N-N-N-N-N-B 3'
MOIETY
CLEAVABLE SITE
BLOCKING

FIG. 4

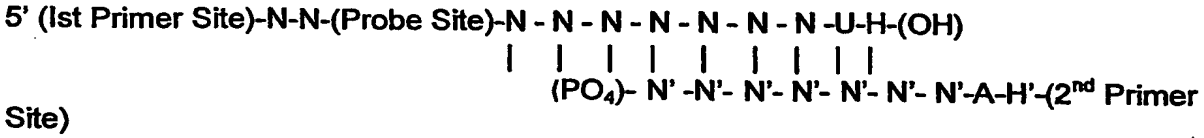
5' (Ist Primer Site)-N-N-(Probe Site)-N - N - N - N - N - N - N -U-H-(OH)
(PO4)- N' -N'- N'- N'- N'- N'- N'-A-H'-(2nd Primer Site)

FIG. 5

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT: WINGER, EDWARD E.
(ii) TITLE OF INVENTION: ACTIVE PROBE AND TARGET DETECTION
(iii) NUMBER OF SEQUENCES: 4
(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: CROSBY HEAFEY ROACH & MAY
(B) STREET: TWO EMBARCADERO CENTER, SUITE 2000
(C) CITY: SAN FRANCISCO
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94111
(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: Windows 95
(D) SOFTWARE: MicrosoftWord 97 / ASCII
(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: 09/602,458
(B) FILING DATE: June 23, 2000
(C) CLASSIFICATION: TO BE ASSIGNED
viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: KOENIG, NATHAN P.
(B) REGISTRATION NUMBER: 38,210
(C) REFERENCE/DOCKET NUMBER: 16231.00340
(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (541) 386-1484
(B) TELEFAX: (415) 391-8269

(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
GCUUCCAUGU CGGCAGAAUG C 21

(2) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
AUUCUGCCCU GAUGAGUCCG CC 22

(2) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
GGCGGACGAA ACAUGGAA 18

(2) INFORMATION FOR SEQ ID NO:4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
GGCGGACGAC ACAUGGAA 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/19915

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C07H 21/04; A61K 48/00; C12Q 1/68; C12N 15/85
US CL : 514/44; 435/6, 325, 375; 536/24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/44; 435/6, 325, 375; 536/24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST, MEDLINE, CAPLUS, EMBASE, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,693,535 (DRAPER et al.) 02 December 1997, see entire document.	1-20
X	USMAN et al. Design, Synthesis, and Function of Therapeutic Hammerhead Ribozymes. Nucleic Acids and Molecular Biology. 1996, vol. 10, pages 243-264, see entire document.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 August 2001 (09.08.2001)	05 NOV 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Andrew Wang Telephone No. 703-308-0196

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